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Anyone for a nice cup of tea? The use of bacterial cellulose for conservation of waterlogged archaeological wood

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ABSTRACT

Current conservation practice is to replace the water in cell structures of waterlogged wood with a synthetic polymer. Because cellulose is an integral component of wood, they are likely to show higher compatibility than synthetic polymers. Bacterial cellulose (BC) can be relatively easily grown from *Acetobacter xylinum* in liquid medium at 25°C–30°C using fructose as nutrition. The purpose of this original research was to determine the potential of growing cellulose directly onto waterlogged wood. Bonding between BC and paper substrates, used to model the cellulose component of wood, was strengthened by using autoclaved media and by optimising access to oxygen. Pre-treating paper with acetone increased bonding strength between BC and Munktell filter paper. Despite the presence of potentially competing bacteria, BC grew at surfaces and within the pores of heavily degraded waterlogged archaeological wood. Initial investigations into conserving waterlogged wood with BC show promise, but require further development.

INTRODUCTION

Kombucha is a probiotic drink produced by fermenting tea using a symbiotic colony of bacteria and yeast. It is reported to improve physical and mental health. Bacteria in kombucha actively ferment sugar to form bacterial cellulose (BC). Bacterial cellulose is an organic compound with the formula $(C_6H_{10}O_5)n$. Although it has an identical chemical formula to plant cellulose, bacterial or microbial cellulose has higher purity, crystallinity of 60% compared with 35%, higher strength, mouldability and increased water content (El Said et al. 2004). BC has microfibrils that are 100 times smaller than those of plant cellulose, and is held together by hydrogen bonding (Esa et al. 2014). The most efficient producer of bacterial cellulose is *Acetobacter xylinum (A. xylinum)* (Jonas and Farah 1998) and it was therefore used in this project. BC can be relatively easily grown from *A. xylinum* in liquid medium at 25°C–30°C with a pH between 3 and 7 and using fructose as a source of nutrition or carbon (Esa et al. 2014). With advances in the ability to synthesize and characterise BC, the material is being used for a wide variety of commercial applications including the production of textiles, cosmetics, and food products, as well as medical applications and paper restoration (Rosa et al. 2011, Santos et al. 2015). However, this article describes the first example of growing BC on a substrate in vitro in the field of conservation.

Waterlogged wood undergoes extensive microbial degradation during burial and conservation aims to dry and stabilize the degraded material. If not conserved properly and allowed simply to dry out in an uncontrolled manner, the cell structures in wood collapse. Current conservation practice at the National Museum of Denmark is to replace the water in the wood cells (and cell walls) with a synthetic polymer, usually polyethylene glycol (PEG), followed by vacuum freeze-drying (Jensen and Strætkvern 2006). The purpose of this preliminary research was to explore the potential and practicalities of using BC as a novel consolidant or bulking agent for conserving waterlogged archaeological wood. Cellulose is an integral component of wood and BC was therefore expected to be more compatible than synthetic polymers. However, effective attachment between BC and wood is considered to be essential both to achieve satisfactory mechanical properties and to disperse stress within the treated wood. Although other researchers have successfully grown BC on bast fibres (Pommet et al.

2008), no attempts to grow BC directly onto other substrates have been published to date.

RATIONALE AND CHALLENGES

As noted above, *A. xylinum* generates cellulose from sugars in aqueous media under ambient conditions. It is a non-pathogenic bacterium and can be relatively easily and safely worked with in the non-sterile conditions most conservation laboratories have available. The overall purpose for this research was to see if cellulose could be grown within the cells of waterlogged archaeological wood. Impregnation of waterlogged wood with a consolidant/bulking agent such as PEG is slow because its rate is controlled by diffusion, the rate of which, in turn, is dependent upon the level of degradation of the wood and the molecular size of the impregnating agent (Jensen 1996). The hypothesis followed in this research was that if the wood could be impregnated with a solution of low-molecular-weight fructose and then inoculated with *A. xylinum*, the bacteria would penetrate the wood, utilise the fructose and produce cellulose in situ. However, *A. xylinum* requires oxygen for respiration and therefore forms cellulosic biofilms mainly at surfaces with access to oxygen (Ruka et al. 2012, Sherif 2014). The fundamental objectives of this research were to explore the following questions: How deep into wood cells can BC grow before the oxygen concentration limits development? If growth of BC in wood can be achieved, can it be successfully dried without collapse/shrinkage? Finally, is it possible to grow BC onto non-sterilised archaeological objects in a non-sterile conservation laboratory?

EXPERIMENTAL APPROACH

Experimental work was divided into three progressive stages. The first stage was to determine whether a pure film of BC could be cultivated in a non-sterile conservation laboratory. The next stage was to grow BC on paper, used as a model cellulosic substrate. Because cellulose is a major component of wood, it was hypothesised that success in growing BC on paper was a good indicator of success in growing BC on wood. The third stage involved applying the most effective techniques to samples of waterlogged wood taken from the Iron Age site of Nydam Mose in Denmark.

METHODS

Cultivation of BC pure film

Standard aseptic microbial techniques were used throughout and the work was carried out in a standard fume cupboard (not in a laminar flow bench), with all media being sterilised in an autoclave at 125°C and 200 kPa for 20 minutes. All tools in contact with cultures were sterilised immediately prior to use with pure ethanol or a combination of ethanol and flaming. Disposable Petri dishes and single-use plastic loops were used. All handling was conducted using nitrile disposable gloves. All chemicals and reagents used were general laboratory grade.

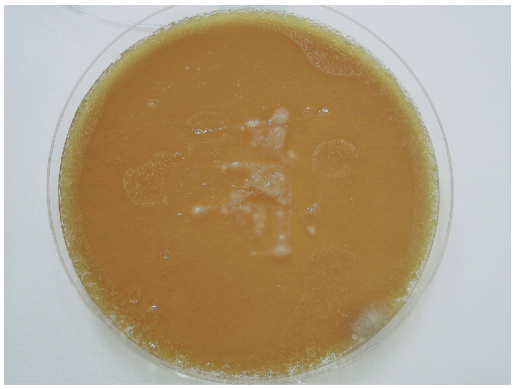

Figure 1. Cultures of *A. xylinum* used were reactivated from their freeze-dried condition by spreading them onto a sterile gel comprising glucose, yeast extract, calcium carbonate, agar and ultrapure water, and allowing them to grow for 1–3 days at ambient temperature

Figure 2. White, translucent BC films formed at surfaces of the medium after 24 hours at ambient temperature

Figure 3. Rinsed and dried BC films were white/cream and tougher than plant cellulose of the same dimensions

Preparation of *A. xylinum* pure culture

The culture of *A. xylinum* used was DSM 15973 *(Komagataeibacter sucrofermentans)*. The bacteria were supplied as a freeze-dried culture in a sterile glass ampoule. These were reactivated by aseptically slurrying them in 5.0 mL of sterile liquid *Gluconobacter oxydans* medium (50 g glucose, 5 g yeast extract, 10 g calcium carbonate, 7.5 g agar and 500 mL ultrapure water with pH adjusted to 6.8 using $CaOH_2$). Following this, 0.5 mL aliquots of the slurry were aseptically spread onto sterile Petri dishes containing solid *Gluconobacter oxydans* media (as previously described, but with 7.5 g agar added). The Petri dishes were incubated at 20°C–25°C until clear signs of bacterial growth were visible, usually after 1 to 3 days (Figure 1).

Fructose medium and growth of BC

Individual colonies of *A. xylinum* were removed from agar plates and added to liquid fructose medium (25 g fructose, 2.5 g yeast extract, 2.5 g peptone, 1.45 g Na_2HPO_4, 0.58 g citric acid and 500 mL ultrapure water). The fructose medium darkened on autoclaving, resulting in brown BC films instead of white. This was attributed to the Maillard reaction, a form of non-enzymatic browning known in baked and fried sugars (Hodge 1953). To avoid this, all medium components, with the exception of fructose, were autoclaved. Fructose was subsequently aseptically added to the medium after cooling to ca. 70°C. Non-sterilised fructose medium was also prepared to see what effect this would have on BC film growth and contamination by microorganisms.

White, translucent BC films formed at surfaces of the medium after 24 hours at ambient temperature, and cohesive films up to 3 mm thick formed after 6–23 days (Figure 2). Literature suggested that the growth of the *A. xylinum* can be halted by immersing the BC films in 0.1 M NaOH (Figure 2) at 80°C for 20 minutes (Toyosaki et al. 1995, Yan et al. 2008). As this treatment was considered hazardous to both user and objects, BC films were instead rinsed repeatedly in ultrapure water. Films were placed on silicone-coated baking parchment and air dried before peeling them off. The resulting films were examined for odour, colour, cohesion and strength (Figure 3).

Growth of BC on model substrate papers

It was hypothesised that success in growing BC on paper was a good indicator of success in growing BC on wood because of the similar cellulose content in both. BC films were grown on three types of paper that comprised a range of surface properties representative both of cultural artefacts and conservation materials. Pure cellulosic Munktell filter paper readily absorbs water and has a micro-corrugated surface profile. Adherence of BC to filter papers was enhanced by pre-treating them for 75 minutes with either acetone or ethanol, and air drying before adding the fructose media. Glossy poster paper and Japanese Kozo paper, frequently used to replace missing areas in books and graphic documents, were also pre-treated before adding the fructose media.

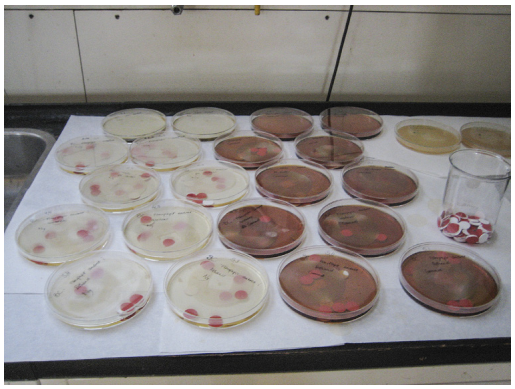

Figure 4. Munktell filter paper was supported on silicone septa in the fructose medium to enhance access to oxygen. The darker BC films to the right of the image were grown from autoclaved fructose media, while the paler films to the left were grown from non-autoclaved media. Darkening was attributed to the Maillard reaction undergone by sugar

Figure 5. Samples of wood from waterlogged wood from Nydam Mose site were impregnated with fructose medium for 6 months before inoculating with *A. xylinum*

Holes were cut in the Munktell filter paper (ca. 2.5 × 2.5 cm) and in the smaller pieces of Kozo and poster papers (ca. 1 × 1 cm respectively) in order to provide areas in which BC could grow.

To provide maximum oxygen access around the various papers, they were supported in the media from below on silicone septa (Figure 4).

Growth of BC on degraded waterlogged archaeological wood

Sections of ash spear shafts from the waterlogged site of Nydam Mose (Gregory and Jensen 2006) were used in these tests. The wood was extensively degraded, with densities as low as 80 kg/cm^3 (fresh ash ca. 670 kg/cm^3). The sections were impregnated with fructose medium for 6 months prior to inoculating with *A. xylinum* simply to ensure the wood was fully impregnated with fructose. In order to determine the presence of fructose within the wood, a test developed in 1964 to identify the presence of fructose in urine – a diagnostic test for diabetes mellitus – was applied (Sarma 1964). Wood samples were cut in half, and KOH pellets placed on the edge and in the centre of the sample using forceps and wearing nitrile rubber gloves and safety glasses to avoid contact with corrosive KOH. The appearance of a red colouration on the pellets after approximately 10 seconds indicated the presence of fructose within the wood structure.

Samples of wood were placed in separate glass containers, covered with fructose media. The containers were inoculated with *A. xylinum* culture and magnetically stirred for 24 hours to maximise the contact between bacteria and wood (Figure 5) before covering with Parafilm, a polyolefin film that allowed access to oxygen while excluding dust particles. After 10 days, thin sections (ca. 50 um) were taken with a razor blade, stained with safranin and examined for evidence of BC using a transmission light microscope (Gregory and Jensen 2006).

RESULTS AND DISCUSSION

Growth of BC in the laboratory

BC films were successfully grown in the non-sterile conservation laboratory using the bacterium *A. xylinum* in a fructose medium. BC in autoclaved media formed initially as gel dots, which grew larger and agglomerated into a film. By contrast, BC grown in non-autoclaved media developed thinner films with finer structure but inferior cohesive properties. The rate of growth was approximately 3–4 times faster in autoclaved media than in non-autoclaved media, around 6 days compared with more than 22. All BC samples grown from autoclaved media, and some from non-autoclaved media, formed double layers of BC. Lower layers were more resistant to breaking when pulled between forceps and therefore stronger and more uniform than the upper layers. The reason for the formation of double layers is unknown and no references to this phenomenon were found in the published literature. Double-layer films were more flexible than single-layer films on drying.

The palest BC films were grown from media where the fructose was first added after autoclaving the other ingredients and where the rate of the Maillard reaction was minimised. Non-autoclaved media resulted in tan-

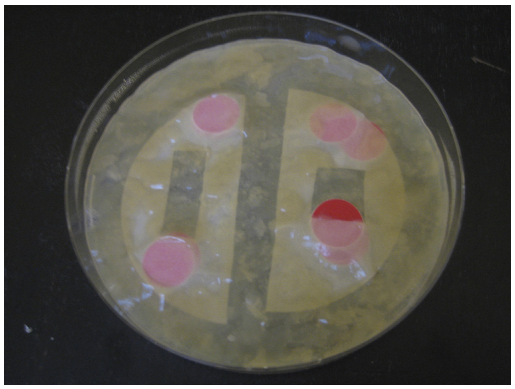

Figure 6. Munktell paper after 23 days of growth in media, where the fructose was added after autoclaving. The paper with the elongated hole (left) was pre-treated with acetone. The paper with the square hole (right) was untreated

Figure 7. Waterlogged archaeological wood showing BC film on the surface of the wood (lower part of wood)

coloured BC films. An odour of residual fructose media and yeast extract was detected in all BC films before and after drying, regardless of the method used to grow them. Rinsing reduced but did not remove odour.

Growth of BC films on model paper substrates

BC attached successfully to Munktell filter paper that had undergone pre-treatment with acetone, where the medium was autoclaved before adding fructose and when paper was placed at the surface of the media thus maximising access to oxygen.

Approximately 15% of the samples showed an attachment between BC and Munktell filter paper sufficiently strong not to be removed by finger pressure, while 50% of the samples where paper was supported by silicone septa at the surface of media showed bonding between paper and BC (Figure 6). The strongest bonding between BC and paper occurred where the latter was pre-treated with acetone and air dried.

No bonding was observed between BC and the poster paper, perhaps due to its highly glossy, low-energy surface and water-repellent properties which likely prevented absorption of medium and attachment of BC. The opposite phenomenon was seen in Kozo paper. BC grew in the holes and encapsulated the Kozo paper to such a high degree that it was not possible to separate the two materials. Films of BC were sufficiently thick as to almost obscure the Kozo paper's surfaces.

Growth of BC on waterlogged archaeological wood

BC film grew on the surfaces of the impregnated Nydam Mose wood, but attached poorly and could readily be scraped off. However, foreign bacteria were evidenced, probably originating from the wood itself. Growth of BC was most effective at surfaces of the impregnated Nydam wood in media, where the fructose was added after autoclaving (Figure 7).

The BC films appeared to reinforce the highly degraded, waterlogged wood surfaces where access to oxygen was high. Because of the high degree of degradation of the waterlogged wood, preparation of thin sections to investigate the presence of BC within the wood cells was difficult. Wood disintegrated on contact with a scalpel or blade. Despite the challenge, at a magnification of 250×, strings of white material resembling BC were visible. The time constraints of this project limited the opportunities to optimise growth of BC within the cells of the wood and further research is needed.

CONCLUSION

Free films of BC were grown successfully in a non-sterile conservation laboratory. Investigations suggested that growth of BC films was optimised in terms of homogeneity, structure, colour and yield when fructose was added after autoclaving the other ingredients in the media, thus inhibiting the Maillard reaction by sugars. Bonding between BC and paper substrates, used to model the cellulose component of wood, was strengthened by using autoclaved media and by optimising access to oxygen. Pre-treating paper with acetone reduced its surface energy and increased bonding strength

between BC and Munktell filter paper. Attachment of BC to Kozo paper was highly successful and paper was almost encapsulated by the rapid growth. BC did not bond satisfactorily to glossy poster paper.

Despite the presence of potentially competing bacteria within the wood from Nydam Mose, BC grew at surfaces and within the pores of heavily degraded waterlogged archaeological wood. These preliminary findings require further investigation, particularly with respect to optimising conditions for growth within wood cells. The availability of oxygen within wood will be a focus for future research, as will the fate of BC on controlled air drying and freeze drying of treated wood.

MATERIALS LIST

Acetobacter xylinum
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